PCT/SG99/00058

\TENT COOPERATION TRE. /

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 31 January 2001 (31.01.01)	
International application No. PCT/SG99/00058	**Applicant's or agent's file reference** GM/MC/R33-68
International filing date (day/month/year) 11 June 1999 (11.06.99)	**Priority date** (day/month/year)
Applicant CHEN, Zhi, Xian et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

15 December 2000 (15.12.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

SG9900058

PATENT COOPERATION TREAT

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

MC/ci

To:
ELLA CHEONG & G.MIRANDAH
P.O.Box 0931
Raffles City 911732
SINGAPORE

13 MAR 2000

Date of mailing *(day/month/year)* 01/03/2000	
Applicant's or agent's file reference GM/MC/R33-68	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/SG 99/00058	International filing date *(day/month/year)* 11/06/1999

Applicant

INSTITUTE OF MOLECULAR AGROBIOLOGY et al.

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sandra De Jong-van Dam

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international pbulication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

MIRANDAH, Gladys
ELLA CHEONG & G. MIRANDAH
P.O. Box 0931
Raffles City
Singapore 911732
SINGAPOUR

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of mailing *(day/month/year)*	07.09.2001
Applicant's or agent's file reference GM/MC/R33-68 151SG38/JKS	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SG99/00058	11/06/1999	11/06/1999

Applicant
INSTITUTE OF MOLECULAR AGROBIOLOGY et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Guerin, A Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GM/MC/R33-68		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SG99/00058	11/06/1999	11/06/1999

International Patent Classification (IPC) or national classification and IPC
C12N15/82

Applicant
INSTITUTE OF MOLECULAR AGROBIOLOGY et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	07.09.2001

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer
Bladier, C
Telephone No. +49 89 2399 7306

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/SG99/00058

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-24 as originally filed

Claims, No.:

1-18 as originally filed

Drawings, sheets:

1 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-18
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Cited documents

1. Reference is made to the following documents:

   D1: WO 97 12512 A (CALGENE INC) 10 April 1997
   D2: GAWEL N. J. *et al.*: PLANT CELL TISSUE AND ORGAN CULTURE, (1990), 23(3), p201-204.

Novelty and inventive step - Articles 33(1)-(3) PCT

2. The subject-matter of **claim 1** is directed to a method for producing a transgenic cotton plant comprising the steps of:
   (a) obtaining cotton petiole explants,
   (b) exposing the petioles explants to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and a selection agent resistance gene to the genome of the cells of the petiole explants,
   (c) culturing the petiole explants to induce callus formation,
   (d) selecting transformed callus that expresses the exogenous gene,
   (e) culturing the selected callus in suspension culture to induce formation of embryoids,
   (f) regenerating the embryoids into whole transgenic cotton plants.

3. Document D1 describes a method for regenerating cotton plants from explant tissue, whereby embryogenic callus is generated directly from petiole explant. This is achieved by maintaining continuously the explant on culture media which do not include any growth regulating hormones (see page 5 lines 5-16 and claims 1, 2). The method can be utilized in the transformation of cotton plants, by:
   (a) obtaining cotton petiole explant (see page 11 lines 7-15, claims 1, 2),
   (b) co-cultivating said explant with *Agrobacterium tumefaciens* comprising an

exogenous gene and a selectable marker, on hormone-free medium to obtain transformed embryogenic callus (see page 12 line 25 to page 17 line 15, claims 4-6)

(c) selecting transformed embryogenic callus that expresses the exogenous gene (see page 18 line 17 to page 19 line 25, particularly page 19 lines 20-22),

(d) (optional) culturing selected embryogenic callus excised from primary callus in suspension tissue culture to obtain embryoids (see page 18 lines 4-8, claim 10),

(e) regenerating the embryoids into whole transgenic cotton plants (see page 18 lines 9-16).

According to the description (see page 10 lines 1-10), the method of the present application differs from method of D1 in that a culture step inducing formation of callus with potency of embryogenesis is done prior to the culture step inducing callus differentiation. However this difference is not apparent in method claim 1 due to the fact that its wording is too broad and imprecise. Step (c) recites 'culturing the petiole explants to induce callus formation'. The term 'callus' encompasses non-embryogenic as well as embryogenic callus and thus step (c) can not be distinguished from step (b) of document D1. Also step (d) of claim 1 that recites 'culturing the selected callus in suspension culture to induce formation of embryoids' can not be distinguished from optional step (d) of document D1 which consists in the culture of excised selected embryogenic callus in suspension tissue culture to induce formation of embryoids. The only difference between method of claim 1 and method of document D1 is that the exposition of the explant to *Agrobacterium tumefaciens* is made simultaneously to the first culture step in D1 (step b) instead of successively in claim 1 (steps b and c). Both possibilities are equivalent and are routine techniques for a person skilled in transformation procedures. Consequently the subject-matter of **claim 1** is novel according to Article 33(2) PCT but can not be considered as involving an inventive step according to Article 33(3) PCT.

It is noted that the broad and vague wording of claim 1 is due to the fact that the steps are defined by means of a result to be achieved rather than in terms of technical features (see also objection item VIII point 6). As a consequence, the intended limitations of said steps are unclear.

4. Document D2 describes a method for obtaining somatic embryos from cotton petiole explants (see Abstract and Materials and Methods), comprising the steps of:

(a) obtaining cotton petiole explants (see Abstract lines 2-3, page 202 left-hand column lines 15-22),

(b) culturing the petiole explants to induce callus formation (see Abstract lines 2-5, page 202 left-hand column lines 22-41),

(c) culturing callus in liquid medium to induce embryoids formation (see Abstract lines 5-8, page 202 left-hand column lines 41-48).

Thus said method differs from method claim 1 in that no transformation and no regeneration step is achieved. Hence document D2 is not prejudicial to the novelty of the subject-matter of claim 1. However it is prejudicial to inventiveness for the following reasons:

- the technical effect associated to a transformation and a regeneration step is to obtain transgenic cotton plants.
- the problem to be solved by the present application may therefore be regarded as providing means to obtain transgenic cotton plants from cotton petiole explants.
- the solution to this problem provided by the application is to add a transformation and a regeneration step to method of document D2.

It is known from document D1 that cells obtained from cotton petiole explant can be transformed by *Agrobacterium tumefaciens* and regenerated in whole transgenic cotton plants. Thus the IPEA is of the opinion that it would be obvious for the person skilled in the art to combine the teaching disclosed in D1 with the method of prior art D2 to solve the aforementioned problem. Consequently the subject-matter of **claim 1** can not be regarded as involving an inventive step in the sense of Article 33(3) PCT.

5. The features of dependent **claims 2-18** are either already known in the prior art (**claims 3-5, 15 and 18** see D2, Materials and Methods lines 23-31; **claims 6, 16 and 17** see D1 page 5 lines 5-16 and claims 1, 2) or fall under the customary practise of a skilled person (**claims 2, 7-14**) and thus do not appear to be suitable if they were combined with claim 1 to render it inventive (Article 33(3) PCT).

Re Item VII

Certain defects in the international application

5. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document D1 is not mentioned in the description, nor is this document identified therein.

Re Item VIII

Certain observations on the international application

6. The steps of method of **claim 1** are not defined in terms of technical features but by means of a result to be achieved ('culturing ... to induce callus formation', 'culturing ... to induce formation of embryoids'). Such definitions are not clear and concise enough to render the subject-matter of said steps distinguishable from the prior art (see objection Item V point 3). In addition according to PCT guidelines III-4.7, claims defined by a result to be achieved are only allowable when they can only be defined in such terms. This is not the case for method claim 1 since it appears possible to characterize steps (c) and (e) in more concrete terms, by including for example the characteristics of the callus and the culture media. Thus an objection is raised according to Article 6 PCT and PCT guidelines III-4.7.

7. There are **contradictions** between **claim 6** and **the description**:
- in the description page 10 lines 1-4, it is written that low concentrations of hormones are important in the callus induction medium (step c) and in the selection medium (step d). However claim 6 recites that the medium of step d does not contain any hormone.
- in the description page 12 lines 13-29, the co-culture medium (step b) and the selection medium (step d) contain 0.05 mg/l 2,4-D and 0.1 mg/l kinetin. However in claim 6, those media do not contain any hormone.

There are also **contradictions in the description itself**:
It is written page 8 lines 23-26 that the improvement of the present invention over previous reported protocols is attributable to several factors. One of these factors is the use of low concentrations of 2,4-D and kinetin in the callus induction medium (step c) (see page 9 lines 4-8) and the selection medium (step d) (see page 10 lines 1-4). **However**, page 10 lines 4-7, it is written that the amount of

2,4-D and kinetin in said steps can be 0 mg/l. This means an absence of hormone and hence a contradiction with the previous mentioned requirement of 'a low concentration of hormone'. Then page 11 lines 5-8, it is written that the media used in the invention are optimized over protocols of the prior art in that low concentrations of hormones are only used at callus initiation stage (step c) and no hormone is used at other stages. Finally page 12 lines 13-29, it appears that media of step b, c and d contain hormones.

These contradictions lead to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

8. It is stated in the description (see summary of the invention page 4 lines 19-29; see page 5 lines 16-20) that the protocol of the application allows higher transformation and regeneration efficiency and a shorter time from transformation to regeneration as compared to previous reported methods. This improvement is attributable to several factors which are (see page 8 line 23 to page 11 lines 1-10):

(a) petioles used as an explant,

(b) glucose as the sole source of carbon except at the young plant growing stage (see page 9 lines 20-27 and page 11 lines 1-4),

(c) media adjusted to pH value of 5.8-7 (see page 9 lines 28-32 and page 11 lines 4-5),

(d) absence of hormones or low concentration of hormones at various stages (page 10 lines 1-10 and page 11 lines 5-8),

(e) a duration of step (e) lower than 20 days (see page 9 lines 9-19),

(f) asparagine and glutamine instead of inorganic ammoniac nitrogen in the medium used for embryoid germination (see page 10 lines 11-21 and page 11 lines 8-31).

Said factors in combinaison are needed to obtain the effect of improved efficiency of transformation and regeneration (see page 8 line 26 and page 9 lines 1-3). Hence they are essential to the definition of the method of the application. Since independent **claim 1** does not contain these features, it does not meet the requirement of Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition

of the invention.

9. The subject-matter of **claim 7** is not supported by the description as required by Article 6 PCT, as its scope is broader than justified by the description. Asparagine or glutamine on their own are used as a source of nitrogen in claim 7. However from the description (see page 11 lines 8-10 and Examples) only asparagine **and** glutamine in combinaison are used to replace inorganic ammoniac nitrogen. No data support that asparagine or glutamine on their own can be used.

10. There is a contradiction between **claim 15** and **dependent claims 16 and 17**. Claim 17 recites that step (c) is carried out in the presence of low concentration of hormone. However dependent claims 16 and 17 recites that 'the concentration ranges from 0 to about .... mg/l'. This means that step (c) can be carried out in absence of hormone. As a result of fact, the subject-matter of those claims is unclear and the dependency of claims 16 and 17 to claim 15 incorrect (Article 6 PCT).

11. There is a contradiction between **claim 12** and **dependent claim 13**. Claim 12 recites that 'culture of step (e) has a duration of less than about 20 days'. However dependent claim 13 recites that 'culture of step (e) has a duration of about 10 days to about 20 days'. This means that step (e) can have a duration of 20 days which is in contradiction with claim 12. As a result of fact, claim 13 can not be dependent to claim 12 (Article 6 PCT).

12. The term 'low concentration' in **claim 15** is vague and inadmissible due to the fact that it characterizes the amount of hormones which is known to be a critical factor in plant regeneration procedures. Thus an objection for lack of clarity is raised (Article 6 PCT).

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
GM/MC/R33-68		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/SG99/00058	11/06/1999	11/06/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant INSTITUTE OF MOLECULAR AGROBIOLOGY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
15/12/2000	07.09.2001
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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/SG99/00058

I. Basis of th report

1. With regard to the **lements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-24 as originally filed

Claims, No.:

1-18 as originally filed

Drawings, sheets:

1 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-18
	No:	Claims	
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-18
Industrial applicability (IA)	Yes:	Claims	1-18
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

R lt m V

R ason d stat m nt under Article 35(2) with r gard to nov lty, inventiv st p or industrial applicability; citations and explanations supporting such statement

Cited documents

1. Reference is made to the following documents:

   D1: WO 97 12512 A (CALGENE INC) 10 April 1997
   D2: GAWEL N. J. *et al.*: PLANT CELL TISSUE AND ORGAN CULTURE, (1990), 23(3), p201-204.

Novelty and inventive step - Articles 33(1)-(3) PCT

2. The subject-matter of **claim 1** is directed to a method for producing a transgenic cotton plant comprising the steps of:
   (a) obtaining cotton petiole explants,
   (b) exposing the petioles explants to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and a selection agent resistance gene to the genome of the cells of the petiole explants,
   (c) culturing the petiole explants to induce callus formation,
   (d) selecting transformed callus that expresses the exogenous gene,
   (e) culturing the selected callus in suspension culture to induce formation of embryoids,
   (f) regenerating the embryoids into whole transgenic cotton plants.

3. Document D1 describes a method for regenerating cotton plants from explant tissue, whereby embryogenic callus is generated directly from petiole explant. This is achieved by maintaining continuously the explant on culture media which do not include any growth regulating hormones (see page 5 lines 5-16 and claims 1, 2). The method can be utilized in the transformation of cotton plants, by:
   (a) obtaining cotton petiole explant (see page 11 lines 7-15, claims 1, 2),
   (b) co-cultivating said explant with *Agrobacterium tumefaciens* comprising an

exogenous gene and a selectable marker, on hormone-free medium to obtain transformed embryogenic callus (see page 12 line 25 to page 17 line 15, claims 4-6)
(c) selecting transformed embryogenic callus that expresses the exogenous gene (see page 18 line 17 to page 19 line 25, particularly page 19 lines 20-22),
(d) (optional) culturing selected embryogenic callus excised from primary callus in suspension tissue culture to obtain embryoids (see page 18 lines 4-8, claim 10),
(e) regenerating the embryoids into whole transgenic cotton plants (see page 18 lines 9-16).

According to the description (see page 10 lines 1-10), the method of the present application differs from method of D1 in that a culture step inducing formation of callus with potency of embryogenesis is done prior to the culture step inducing callus differentiation. However this difference is not apparent in method claim 1 due to the fact that its wording is too broad and imprecise. Step (c) recites 'culturing the petiole explants to induce callus formation'. The term 'callus' encompasses non-embryogenic as well as embryogenic callus and thus step (c) can not be distinguished from step (b) of document D1. Also step (d) of claim 1 that recites 'culturing the selected callus in suspension culture to induce formation of embryoids' can not be distinguished from optional step (d) of document D1 which consists in the culture of excised selected embryogenic callus in suspension tissue culture to induce formation of embryoids. The only difference between method of claim 1 and method of document D1 is that the exposition of the explant to *Agrobacterium tumefaciens* is made simultaneously to the first culture step in D1 (step b) instead of successively in claim 1 (steps b and c). Both possibilities are equivalent and are routine techniques for a person skilled in transformation procedures. Consequently the subject-matter of **claim 1** is novel according to Article 33(2) PCT but can not be considered as involving an inventive step according to Article 33(3) PCT.

It is noted that the broad and vague wording of claim 1 is due to the fact that the steps are defined by means of a result to be achieved rather than in terms of technical features (see also objection item VIII point 6). As a consequence, the intended limitations of said steps are unclear.

4. Document D2 describes a method for obtaining somatic embryos from cotton petiole explants (see Abstract and Materials and Methods), comprising the steps of:
(a) obtaining cotton petiole explants (see Abstract lines 2-3, page 202 left-hand column lines 15-22),
(b) culturing the petiole explants to induce callus formation (see Abstract lines 2-5, page 202 left-hand column lines 22-41),
(c) culturing callus in liquid medium to induce embryoids formation (see Abstract lines 5-8, page 202 left-hand column lines 41-48).

Thus said method differs from method claim 1 in that no transformation and no regeneration step is achieved. Hence document D2 is not prejudicial to the novelty of the subject-matter of claim 1. However it is prejudicial to inventiveness for the following reasons:
- the technical effect associated to a transformation and a regeneration step is to obtain transgenic cotton plants.
- the problem to be solved by the present application may therefore be regarded as providing means to obtain transgenic cotton plants from cotton petiole explants.
- the solution to this problem provided by the application is to add a transformation and a regeneration step to method of document D2.
It is known from document D1 that cells obtained from cotton petiole explant can be transformed by *Agrobacterium tumefaciens* and regenerated in whole transgenic cotton plants. Thus the IPEA is of the opinion that it would be obvious for the person skilled in the art to combine the teaching disclosed in D1 with the method of prior art D2 to solve the aforementioned problem. Consequently the subject-matter of **claim 1** can not be regarded as involving an inventive step in the sense of Article 33(3) PCT.

5. The features of dependent **claims 2-18** are either already known in the prior art (**claims 3-5, 15 and 18** see D2, Materials and Methods lines 23-31; **claims 6, 16 and 17** see D1 page 5 lines 5-16 and claims 1, 2) or fall under the customary practise of a skilled person (**claims 2, 7-14**) and thus do not appear to be suitable if they were combined with claim 1 to render it inventive (Article 33(3) PCT).

Re Item VII

Certain d fects in the international application

5. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the document D1 is not mentioned in the description, nor is this document identified therein.

Re Item VIII

Certain observations on the international application

6. The steps of method of **claim 1** are not defined in terms of technical features but by means of a result to be achieved ('culturing ... to induce callus formation', 'culturing ... to induce formation of embryoids'). Such definitions are not clear and concise enough to render the subject-matter of said steps distinguishable from the prior art (see objection Item V point 3). In addition according to PCT guidelines III-4.7, claims defined by a result to be achieved are only allowable when they can only be defined in such terms. This is not the case for method claim 1 since it appears possible to characterize steps (c) and (e) in more concrete terms, by including for example the characteristics of the callus and the culture media. Thus an objection is raised according to Article 6 PCT and PCT guidelines III-4.7.

7. There are **contradictions** between **claim 6** and **the description**:
- in the description page 10 lines 1-4, it is written that low concentrations of hormones are important in the callus induction medium (step c) and in the selection medium (step d). However claim 6 recites that the medium of step d does not contain any hormone.
- in the description page 12 lines 13-29, the co-culture medium (step b) and the selection medium (step d) contain 0.05 mg/l 2,4-D and 0.1 mg/l kinetin. However in claim 6, those media do not contain any hormone.

There are also **contradictions in the description itself**:
It is written page 8 lines 23-26 that the improvement of the present invention over previous reported protocols is attributable to several factors. One of these factors is the use of low concentrations of 2,4-D and kinetin in the callus induction medium (step c) (see page 9 lines 4-8) and the selection medium (step d) (see page 10 lines 1-4). **However**, page 10 lines 4-7, it is written that the amount of

2,4-D and kinetin in said steps can be 0 mg/l. This means an absence of hormone and hence a contradiction with the previous mentioned requirement of 'a low concentration of hormone'. Then page 11 lines 5-8, it is written that the media used in the invention are optimized over protocols of the prior art in that low concentrations of hormones are only used at callus initiation stage (step c) and no hormone is used at other stages. Finally page 12 lines 13-29, it appears that media of step b, c and d contain hormones.

These contradictions lead to doubt concerning the matter for which protection is sought, thereby rendering the claims unclear (Article 6 PCT).

8. It is stated in the description (see summary of the invention page 4 lines 19-29; see page 5 lines 16-20) that the protocol of the application allows higher transformation and regeneration efficiency and a shorter time from transformation to regeneration as compared to previous reported methods. This improvement is attributable to several factors which are (see page 8 line 23 to page 11 lines 1-10):

(a) petioles used as an explant,

(b) glucose as the sole source of carbon except at the young plant growing stage (see page 9 lines 20-27 and page 11 lines 1-4),

(c) media adjusted to pH value of 5.8-7 (see page 9 lines 28-32 and page 11 lines 4-5),

(d) absence of hormones or low concentration of hormones at various stages (page 10 lines 1-10 and page 11 lines 5-8),

(e) a duration of step (e) lower than 20 days (see page 9 lines 9-19),

(f) asparagine and glutamine instead of inorganic ammoniac nitrogen in the medium used for embryoid germination (see page 10 lines 11-21 and page 11 lines 8-31).

Said factors in combinaison are needed to obtain the effect of improved efficiency of transformation and regeneration (see page 8 line 26 and page 9 lines 1-3). Hence they are essential to the definition of the method of the application. Since independent **claim 1** does not contain these features, it does not meet the requirement of Article 6 PCT taken in combination with Rule 6.3(b) PCT that any independent claim must contain all the technical features essential to the definition

of the invention.

9. The subject-matter of **claim 7** is not supported by the description as required by Article 6 PCT, as its scope is broader than justified by the description. Asparagine or glutamine on their own are used as a source of nitrogen in claim 7. However from the description (see page 11 lines 8-10 and Examples) only asparagine **and** glutamine in combinaison are used to replace inorganic ammoniac nitrogen. No data support that asparagine or glutamine on their own can be used.

10. There is a contradiction between **claim 15** and **dependent claims 16 and 17**. Claim 17 recites that step (c) is carried out in the presence of low concentration of hormone. However dependent claims 16 and 17 recites that 'the concentration ranges from 0 to about .... mg/l'. This means that step (c) can be carried out in absence of hormone. As a result of fact, the subject-matter of those claims is unclear and the dependency of claims 16 and 17 to claim 15 incorrect (Article 6 PCT).

11. There is a contradiction between **claim 12** and **dependent claim 13**. Claim 12 recites that 'culture of step (e) has a duration of less than about 20 days'. However dependent claim 13 recites that 'culture of step (e) has a duration of about 10 days to about 20 days'. This means that step (e) can have a duration of 20 days which is in contradiction with claim 12. As a result of fact, claim 13 can not be dependent to claim 12 (Article 6 PCT).

12. The term 'low concentration' in **claim 15** is vague and inadmissible due to the fact that it characterizes the amount of hormones which is known to be a critical factor in plant regeneration procedures. Thus an objection for lack of clarity is raised (Article 6 PCT).

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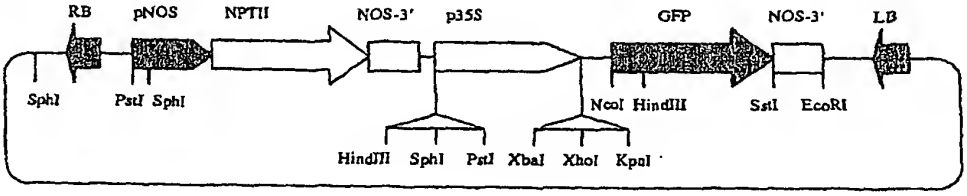
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: HIGH-EFFICIENCY *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF COTTON USING PETIOLE EXPLANTS



(57) Abstract: A method is disclosed for producing a transgenic cotton plant by *Agrobacterium*-mediated transformation of petiole tissue. The method comprises the steps of (a) obtaining cotton petiole explants, (b) exposing the petiole explants to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the petiole explant, (c) culturing the petiole explants to induce callus formation, (d) selecting transformed callus that expresses the exogenous gene, (e) culturing the selected callus in suspension culture to induce formation of embryoids, (f) regenerating the embryoids into whole transgenic cotton plants.

WO 00/77230 A1

High-Efficiency *Agrobacterium*-mediated Transformation of Cotton Using Petiole Explants

Technical Field

The present invention relates to the general field of genetic engineering of plants, in particular to the introduction of exogenous genetic material into cotton by *Agrobacterium* transformation of cotton petiole explants followed by somatic embryo regeneration.

Background

Cotton is one of the most valuable and widely grown cash crops internationally. Its annual production worldwide is over 100 million bales valued at US$45 billion. Asia is the biggest cotton production area, with four out of five world top cotton producers located in this region. Cotton is not only the main supporter for the textile industry, but it also provides a huge and profitable market for manufacturers of chemicals for weed, disease and pest control. There are diverse opportunities for cotton molecular improvement, including improvement of yield and fiber quality and creation of new varieties that are resistant to herbicides, insects, nematodes and diseases (Steward, 1991).

Tissue Culture of Cotton: In 1935, Skovsted reported the first embryo culture of cotton. Beasley

(1971) reported callus formation in cotton as an outgrowth from the micropylar end of fertilized ovules on Murashige & Skoog (MS) medium. Somatic embryogenesis was achieved from a suspension culture of *G. klotzschianum* (Price & Smith, 1979). In 1983, Davidonis & Hamilton first succeeded in efficient and repeatable regeneration of cotton (*G. hirsutum* L.) plants from callus after two-year cultivation. Cotton plants were since regenerated through somatic embryogenesis from different explants (Zhang & Feng, 1992; Zhang, 1994) including cotyledon (Davidonis et al., 1987; Davidonis & Hamilton, 1983; Finer, 1988; Firoozabady et al., 1987), hypocotyl (Cousins et al., 1991; Rangan & Zavala, 1984; Rangan & Rajasekaran, 1996; Trolinder & Goodin, 1988; Umbeck et al., 1987, 1989), stem (Altman et al., 1990; Bajaj et al., 1989; Chen, et al. 1987; Finer & Smith, 1984), shoot apex (Bajaj et al., 1985; Gould et al., 1991; Turaev & Shamina, 1986), immature embryo (Beasley, 1971; Stewart & Hsu, 1977, 1978), petiole (Finer & Smith, 1984; Gawel et al., 1986; Gawel & Robacker, 1990), leaf (Finer & Smith, 1984; Gawel & Robacker, 1986), root (Chen & Xia, 1991; Kuo et al., 1989), callus (Finer & McMullen, 1990; Trolinder et al., 1991) and protoplast (Chen et al., 1989).

Transformation of cotton: *Agrobacterium*-mediated cotton transformation was first reported a decade ago with hypocotyl and cotyledon as explants (Firoozababy et al., 1987; Umbeck et al., 1987). Several useful genes have been introduced into cotton via *Agrobacterium*-mediated transformation, including insect and herbicide resistance genes (Perlak et al., 1990; Trolinder et al., 1991; Chen et al., 1994). Explants

(such as hypocotyl, cotyledon, callus generat d from hypocotyl and cotyledon, as w ll as immature embryos) have been used for *Agrobacterium*-mediated transformation and particle bombardment (de Framond et al., 1983; Finer & McMullen, 1990; Firoozabady et al., 1987; Perlak et al., 1990; Rangan & Rajasekaran, 1996; Rajasekaran et al., 1996; Trolinder et al., 1991; Umbeck et al., 1987, 1989, 1992). In addition, meristematic tissue of excised embryonic axes has also been used for cotton transformation by particle bombardment (Chlan et al., 1995; John, 1996; John & Keller, 1996; McCabe & Martinell, 1993). Zhou et al. (1983) transformed cotton by injecting DNA into the axile placenta one day after self-pollination.

However, the transformation rates were generally low, ranging from 20 to 30% when hypocotyl were used as explant (Firoozababy et al., 1987; Cousins et al., 1991; Rajasekaran et al., 1996). A significantly higher transformation efficiency, up to 80%, was reported when cotyledon was used as explant and the ocs gene encoding octopine synthetase used as the reporter gene (Firoozababy et al., 1987). However, the validity of octopine as a marker for transformation is questionable because octopine has been found in several plant species certainly not transformed by infection with *A. tumefaciens* (Wendt-Gallitelli and Dobrigkeit, 1973). A more recent report indicated that the transformation efficiency of cotyledon was about 20 to 30% (Cousins et al., 1991). The transformation efficiency was even lower when particle bombardment method was used (Keller et al., 1997). A difference in the type of explants used for transformation could have a significant effect on the efficiency of

transformation and regeneration. It has been reported, for example, that for reducing false positive transformants, cotyledon was a better explant than hypocotyledon (Firoozabady et al., 1987).

Cotton transformation also is highly dependent on genotype (Trolinder, 1985a, 1986; Trolinder & Goodin, 1987, 1988a, 1988b; Trolinder & Chen, 1989). Apart from a few cultivars which are regenerable and transformable, such as *Gossypium hirsutum* cv. Coker 312 and *G. hirsutum* Jin 7, most other important elite commercial cultivars, such as *G. hirsutum* cv. D&P 5415 and *G. hirsutum* cv. Zhongmian 12, are not regeneratable and transformable by these methods. The absence of a high-efficiency plant regeneration method has been regarded as a major obstacle to the application of *Agrobacterium*-mediated transformation to cotton (Gawel et al., 1986; Firoozabady et al., 1987).

Summary of the Invention

To overcome the problems associated with previously reported methods, an efficient transformation procedure using petiole as an explant has been developed, along with a set of correspondingly improved media. This method provides several advantages in comparison to the hypocotyl and cotyledon methods: (1) explants are easy to obtain; (2) transformation efficiency is higher; (3) *Agrobacterium* contamination is very rare; (4) efficiency in regeneration is higher; and (5) the time from transformation to regeneration of plantlets is reduced. Two cotton varieties, i.e. Coker 312 and Si-Mian 3, have been successfully transformed with this method, and more than 30 independent transgenic lines from

Coker 312 showing strong activity of the marker transgene have been obtained. This method is applicable to other cotton varieties such as Jin 7 and Ji 713 from China, Siokra 1-3 from Australia, T25, Coker 201 and Coker 310 from the U.S.A.

Brief Description of the Figure

Figure 1 shows the plasmid pBI121GFP, containing GFP as the reporter gene and the NPT II (neomycin phosphotransferase) gene as a selectable marker, used for *Agrobacterium*-mediated transformation of cotton petiole according to the methods of the present invention.

Detailed Description

An efficient method is disclosed for genetic transformation of cotton plants, including elite lines, using cotton petiole as an explant. By using petiole explants, plus a set of improved media, transformation efficiency is significantly enhanced and the time required from transformation to regeneration is shortened in comparison to previously reported methods.

By using the methods of the present invention, the whole process from *Agrobacterium* transformation to the regeneration of transgenic plantlets can take about 6-7 months. The reported hypocotyl and cotyledon methods usually required 7-9 months or longer to complete the same process (Cousins et al., 1991; Chen et al., unreported observation). Another two months were required for growing the small plantlets to a suitable size for potting in soil.

Techniques for introducing exogenous genes into *Agrobacterium* such that they will be transferred stably

to a plant or plant tissue exposed to the *Agrobacterium* are well-known in the art and do not form part of the present invention. It is advantageous to use a so-called "disarmed" strain of *Agrobacterium* or Ti plasmid, that is, a strain or plasmid wherein the genes responsible for the formation of the tumor characteristic of the crown gall disease caused by wild-type *Agrobacterium* are removed or deactivated. Numerous examples of disarmed *Agrobacterium* strains are found in the literature (*e.g.*, pAL4404, pEHA101 and pEH 105 (Walkerpeach & Veltern, 1994)). It is further advantageous to use a so-called binary vector system, such as that described in Schilperoort et al., 1990, 1995. A binary vector system allows for manipulation in *E. coli* of the plasmid carrying the exogenous gene to be introduced into the plant, making the process of vector construction much easier to carry out.

Similarly, vector construction, including the construction of chimeric genes comprising the exogenous gene that one desires to introduce into the plant, can be carried out using techniques well-known in the art and does not form part of the present invention. Chimeric genes should comprise promoters that have activity in the host in which expression is desired. For example, it is advantageous to have a series of selectable markers for selection of transformed cells at various stages in the transformation process. A selectable marker (for example a gene conferring resistance to an antibiotic such as kanamycin, cefotaxime or streptomycin) linked to a promoter active in bacteria would permit selection of bacteria containing the marker (*i.e.*, transformants). Another selectable marker linked to a plant-active promoter,

such as the CaMV 35S promoter or a T-DNA promoter such as the NPT II NOS promoter, would allow selection of transformed plant cells. The exogenous gene that is desired to be introduced into the plant cell should comprise a plant-active promoter in functional relation to the coding sequence, so that the promoter drives expression of the gene in the transformed plant. Again, plant-active promoters, such as the CaMV 35S, the NPT II NOS promoter or any of a number of tissue-specific promoters, are well-known in the art and selection of an appropriate promoter is well within the ordinary skill in the art.

The present method can be used to produce transgenic plants expressing any number of exogenous genes, and is not limited by the choice of such a gene. The selection of the desired exogenous gene depends on the goal of the researcher, and numerous examples of desirable genes that could be used with the present invention are known in the art (e.g., the family of *Bacillus thuringiensis* toxin genes, herbicide resistance genes such as shikimate synthase genes that confer glyphosate resistance, U.S. Patent No. 5,188,642, or a 2,4-D monooxygenase gene that confers resistance to 2,4-dichlorophenoxyacetic acid (2,4-D), Bayley et al., Theoretical and Applied Genetics, vol. 82, pp. 645-49, male sterility genes such as the antisense genes of U.S. Patent No. 5,741,684 (Fabijanski, et al.), or even the elaborate crop protection systems described in U.S. Patent No. 5,723,765 (Oliver et al.)).

Cotton regeneration is considered in the art to be heavily variety-dependant. The Coker series of cotton varieties have been shown to be relatively easy to

transform. However, DP 5412, Zhongmain 12 and many other varieties still have difficulties associated with regeneration. The situation is the same for *G. barbadense* and other diploid species. While somatic embryogenesis and regeneration of whole plants is a highly genotype-dependent process in cotton, successful transformation and regeneration of two distinct cotton varieties, *i.e.* Coker 312 from U.S.A. and Si-Mian 3 from China, has been demonstrated using the methods of the present invention. It this therefor believed that the present invention has wide applicability to transformation of a variety of cotton lines.

Transgene integration in the genome of cotton produced by the methods of the present invention was confirmed using standard Southern hybridization techniques, as can identification of the copy number of the inserted transgene in each transgenic line (see Example 6, below). The F1 generation of transgenic cotton can be tested for the presence of the transgene, and inheritance pattern of the transgene in the F1 generation can be analyzed to confirm stability and inheritability.

As compared with other reported protocols, the cotton transformation system of the present invention has higher transformation efficiency and survival rate. This is attributable to several factors. In the present invention, petiole was used as an explant for transformation. Different types of cotton explants can have significant effects on the efficiencies of plant transformation and regeneration (Firoozabady et al., 1987). Induction of somatic embryogenesis from petiole was reported previously. But regeneration was either unsuccessful or very poor (Finer and Smith, 1984; Gawel

et al., 1986). With the present invention, the efficiency of regeneration was significantly improved by using the improved media discussed below. In a preferred embodiment, calli of high quality were obtained when tender petioles rich in parenchyma cell in primary vascular bundle tissue were cultured in the MMSI medium (described below) with low concentrations of 2,4-D and Kinetin.

With the present invention, the time for embryo induction in suspension culture can be shortened to 10 - 14 days, from a previously reported 3 weeks (Cousins et al., 1991). It was found that a shortened period of suspension culture treatment is important for high frequency induction of embryogenesis. It is also important for reducing production of abnormal embryos, since a high percentage of vitreous embryos that are poor in regeneration are produced when cotton calli are maintained in suspension culture for too long (Chen et al, unpublished observation).

For maximum cell growth at different stages except at the young plant growing stage, glucose was used as the sole carbon source. The amount of glucose in the media can be from about 10 to about 50 g/l, preferably about 30 g/l. At the young plant growing stage, glucose and sucrose at about 10 g/l respectively as carbon sources are preferable for promotion of healthy plantlets growth.

For growth of callus, embryogenesis and callus proliferation, pH range can be from 5.8 to 7.5, preferably pH 6.2 - 7.0, most preferably at pH 6.5. A medium of pH 7.0 is preferable for healthy root growth of plantlets.

For effective callus initiation and induction of the potency of embryogenesis, low concentrations of 2,4-D and kinetin in the callus induction and selection medium is important. The amount of 2,4-D can be from 0 to about 0.5 mg/l, preferably about 0.05 mg/l. The amount of kinetin can be from 0.0 mg/l to about 1.0 mg/l, preferably about 0.1 mg/l. In the callus differentiation stage and embryoid germination stage, best result were obtained when no plant hormone was added to the media.

The amino acids asparagine and glutamine are better nitrogen sources than inorganic ammonia nitrogen for specifically supporting embryoids germination and root development. In the embryoid germination medium, the amount of asparagine can be about 200 to about 1000 mg/l, preferably about 500 mg/l. The amount of glutamine can be about 500 to about 2000 mg/l, preferably about 1000 mg/l. With these optimized nitrogen sources, the growth of non-embryogenic calli was inhibited while the germination, growth and root development of embryoids were preferentially promoted.

At different stages of cotton transformation except co-culture with Agrobacterium, plant tissue and callus are preferably maintained at 28°C but can be varied from 25-35°C. For effective transformation, temperature in co-culture stage should not be higher than 28°C. A light condition of 16 hrs. light (60-90, $\mu Em^{-2}S^{-1}$) and 8 hrs. dark per day is preferable for all stages of cotton transformation and regeneration.

Unlike previously reported transformation and regeneration protocols (Umbeck et al., 1987; Firoozabady et al., 1987, Cousins et al.), the media used in the present invention are optimized in several

respects: (a) glucose is used as a sole carbon source in all culture media except in the medium used to culture young plants previous to planting out in the greenhouse; (b) the media is adjusted to higher pH value (6.5-7.0); (c) lower concentration of 2,4-D (0.05mg/l) and kinetin (0.1 mg/l) is used only at callus initiation stage, no hormone is used at other stages; (d) asparagine and glutamine are used to replace inorganic ammoniac nitrogen in the medium used for embryoid germination. These modifications are adapted for the physiological requirement of cotton embryoid development and plantlet growth. It has been found that healthy embryoid development and plantlet growth, especially root system development, are largely attributable to these optimized media. For example, it has been found that asparagine and glutamine were better nitrogen source than inorganic ammonia nitrogen for supporting embryoid germination and root development. In the preferred MMS3 medium (described below), which contains asparagine and glutamine as the nitrogen source, the growth of non-embryogenic calli was inhibited while the germination, growth and root development of embryoids were preferentially promoted. Because of the healthy root development, the survival rate of potted transgenic cotton plants obtained by the methods of the present invention is almost 100%. With the reported hypocotyl and cotyledon protocols (Umbeck et al., 1987; Firoozabady et al., 1987), poor root development has been regarded as the main reason accounting for poor survival rate of potted transgenic cotton plants.

The following are preferred plant tissue culture media used in the Examples:

(1) Seedling growing medium (per liter):
½ MS basal salt mixture (Sigma M5524)
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 7.0

(2) Petiole pre-culture medium (per liter):
MS basal salt mixture
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 7.0

(3) Co-culture medium (per liter):
MS basal salt mixture
10 mg Thiamine-HCl
1 mg Pyridoxine-HCl
1 mg Nicotinic acid
100 mg Myo-inositol
0.05 mg 2,4-dichlorophenoxyacetic acid (2,4-D)
0.1 mg Kinetin
30 g Glucose
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 6.5

(4) MMS1 - callus induction and selection medium (per liter):
Co-culture medium
50 mg Kanamycin
500 mg Cefotaxime

(5) MMS2 - differentiation medium (per litre):

MS basal salt mixture
10 mg Thiamin -HCl
1 mg Pyridoxine-HCl
1 mg Nicotinic acid
100 mg Myo-inositol
1.9 g KNO_3
30 g Glucose
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 6.5

(5) MMS3 - embryoid germination medium (per litre):

3.8 g KNO_3
440 mg $CaCl_2 \cdot H_2O$
375 mg $MgSO_4 \cdot 7H_2O$
170 mg KH_2PO_4
1 g Glutamine
500 mg Asparagine
43 mg EDTA ferric-Na salt
MS micronutrients (Murashige and Skoog, 1962)
10 mg Thiamine-HCl
1 mg Pyridoxine-HCl
1 mg Nicotinic acid
100 mg Myo-inositol
30 g Glucose
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 6.5

(7) Young plant growing medium

S&H medium Macro and Micro elements (Strewart and Hsu, 1977)

10 mg Thiamine-HCl
1 mg Pyridoxine-HCl
1 mg Nicotinic acid
100 mg Myo-inositol
10 g Glucose
10 g Sucrose
0.9 g $MgCl_2 \cdot 6H_2O$
2.0 g gellan gum (Phytagel™, Sigma)
pH 7.0

The following Examples are intended to illustrate the present invention, and not in any way to limit its scope, which is solely defined by the claims.

Example 1: *Agrobacterium* strain and plasmids

A. tumefaciens strain LBA 4404 (pBI121GFP) was used for transformation of cotton petiole and young stem. The physical map of pBI121GFP is shown in Fig.1, which contains GFP as a reporter gene and NPTII gene (encoding neomycin phosphotransferase) as a selectable marker. The GFP and NPTII genes are under the control of CaMV 35S promoter and nos promoter respectively. For construction of pBI121GFP, a 720 bp XbaI-SstI fragment of GFP gene from the pGFP2 plasmid (from Dr. N. H. Chua, Rockefeller University, New York) was cloned into the same sites in plasmid vector pBI121 (Clontech) to replace the GUS gene. The pBI121GFP plasmid was introduced into *A. tumefaciens* LBA 4404 by electroporation.

Example 2: Plant material

Upland cotton varieties Coker 312 from the U.S.A. and Si-Mian 3 from Shanxi Cotton Research Institute in China were used in the experiments.

Tender petioles were collected from plants 8-12 weeks old grown in a greenhouse with low light conditions. The petioles were surface-sterilized with 70% ethanol for a few seconds, followed by 20% bleach solution (Clorox Co. USA, 1% available chlorine) for 20 min. After rinsing five times in sterilized water, the petioles were pre-cultured in MS medium for 3 days.

Example 3: Plant transformation

A single colony of *A. tumefaciens* strain LBA 4404 (pBI121GFP) was inoculated in liquid LB medium with 50 mg/L Rifampicin, 50 mg/L kanamycin and 100 mg/L streptomycin. The bacteria was grown overnight at 28°C in a shaker of 200 rpm. The bacterium cultures were diluted using liquid MS medium to OD600 = 0.3.

The petiole and young stem were cut into about 2 cm long segments. The segments were soaked in the diluted bacterium suspension for 5 min, then transferred onto plastic plates (100 x 25 mm) containing a filter paper soaked in 50 ml of co-culture medium. The plates were kept in an incubator of 24°C under continuous light for 48 hrs. The co-cultured explants were transferred onto MMS1 medium and incubated at 28°C with 16 hrs light (60-90 , $\mu Em^{-2}s^{-1}$) and 8 hrs dark per day. After 2-4 weeks calli were initiated at the cut ends of petiole segments. After 4-6 weeks kanamycin resistant calli had appeared, and the number of calli were counted and the expression of GFP gene was examined.

Under the fluorescence microscope, the untransformed control callus appeared red in colour, while the transformed callus expressing GFP gene displayed distinct green fluorescence. A total of 113 putative transformed calli were examined for GFP activity, the transformation frequency of GFP gene was 39.8% (Table 1). When petioles from cotton variety Si-Mian 3 were used for transformation, 11 calli were found GFP positive from 26 calli tested, transformation efficiency was 42.3%.

Table 1: Transformation frequencies of petioles from cotton Coker 312 and Si-Main 3

Varieties	Number of calli tested	Number of GFP positive calli	GFP gene transformation frequency (%)
Coker 312	113	45	39.8
Si-Mian 3	26	11	42.3

Example 4: Induction of somatic embryogenesis and plant regeneration

The calli with vigorous growth and strong expression of GFP were selected and transferred into liquid MMS2 medium for suspension culture for 2 weeks. Friable cream-colored granular calli were selected and transferred to semi-solid differential medium, MMS2. After about 2 months a large number of embryoids were produced. Cytoplasmic dense embryogenic structures were gradually developed and large embryos were produced on the medium within 1-2 month. A short time of suspension culture treatment was very important, not only for high frequencies of embryogenesis induction, but also for production of embryoids of good quality.

Expression of GFP gene was checked again and all were GFP positive.

The embryoids and embryogenic calli with strong GFP activity were transferred onto the MMS3 medium. After 1-2 months the plantlets that were about 1-2 cm in height with 1-2 true leaves and good root development were transferred to the Young Plant Growing Medium for about one month. About one month later, young plants with 6-8 leaves and about 10-15 cm in height were potted in soil and move to the glasshouse. All 30 potted transgenic plants survived and were found expressing GFP protein. The total time required to obtain transgenic plantlets using was under 7 months, and plantlets were reading for potting out in the greenhouse in about 2 additional months (see Table 2).

Table 2: The time frame from transformation of petiole segments to plant regeneration (Coker 312)

Transform-ation	Callus obtained	Embryos appeared	Regener-ation	Plants planted to groen house	Flowering
10/4/98	26/5/98	29/7/98	1/11/98	31/12/98	14/2/99

Example 5: Detection of GFP Protein Activity

The expression of GFP protein activity was detected using a Leica MZ FLIII Fluorescence stereo microscope with a 480/40 nM excitation filter and a 510 nM barrier filter.

Green fluorescence of GFP gene can be easily distinguished in the transformed callus, embryoids, and young plantlets, with the untransformed control appeared red in colour under the fluorescence Stereo microscope. The exceptions were the untransformed

roots, which appeared dim green under the fluorescence microscope, probably due to some chromophorous chemicals accumulated in roots. But the roots with GFP activity could still be identified because the green fluorescence produced by GFP protein was brighter and appeared more uniform. Under the blue light produced by the fluorescence stereo microscope, red fluorescence is clearly visible in untransformed green plant tissues that are enriched with chlorophyll such as leaf and stem. In GFP-positive green plant tissues, yellow fluorescence also was detected because of the overlapping of red and green fluorescence. However, the expression of GFP gene in petal and anther was poorer in comparison to that in other parts of plant.

Example 6: Analysis of Transgenic Plants

Genomic DNA from putatively transformed lines and non-transformed control plants was purified according to Paterson et al. (1993). After digestion with *EcoRI*, which cuts inj-between left border of T-DNA and Nos-3' terminator of the chimerical GFP gene (Fig. 1), DNA was separated on a 0.8% TAE agarose gel and transferred to Hybond-N membrane according to manufacturer's instructions. DNA was fixed to the membrane by UV crossing linking before hybridizing to the DIG labeled coding region of the GFP gene. Hybridized probe was detected with anti-DIG-AP conjugate according to manufacturer's instructions (BOEHRINGER MANNHEIM).

The genomic DNA samples from 11 randomly selected transgenic lines and 1 untransformed control plant were analyzed Southern hybridization, using the coding region of GFP gene as the hybridization probe. The data indicate that 7 out of 11 lines have a single

copy, 3 lines have 2 copies, and 1 line has 6 copies of T-DNA insertion. The high percentage of transgenic lines with a single copy of T-DNA insertion suggests that this transformation protocol has less risk of gene silencing and undesirable insertion mutants.

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We claim:

1. A method for producing a transgenic cotton plant comprising the steps of:

(a) obtaining cotton petiole explants,

(b) exposing the petiole explants to a culture of *Agrobacterium tumefaciens* that harbors a vector comprising an exogenous gene and a selectable marker, the *Agrobacterium* being capable of effecting the stable transfer of the exogenous gene and selection agent resistance gene to the genome of the cells of the petiole explant,

(c) culturing the petiole explants to induce callus formation,

(d) selecting transformed callus that expresses the exogenous gene,

(e) culturing the selected callus in suspension culture to induce formation of embryoids,

(f) regenerating the embryoids into whole transgenic cotton plants.

2. The method of claim 1 wherein the petiole explants are pre-cultured for a period of time prior to exposure to the culture of *Agrobacterium tumefaciens*.

3. The method of claims 1 wherein the culture media used in steps (b)-(e) have glucose as the sole carbon source.

4. The method of claim 3 wherein the glucose is in an amount of about 10 g/l to about 50 g/l.

5. The method of claim 4 wherein the glucose is in an amount of about 30 g/l.

6. The method of claim 1 wherein the culture media used in steps (b) and (d)-(f) do not contain hormones.

7. The method of claim 1 wherein embyroid germination is carried out in a medium having a source of nitrogen selected from the group consisting of asparagine, glutamine or both asparagine and glutamine.

8. The method of claim 7 wherein the source of nitrogen is in an amount of about 700 mg/l to about 5 g/l.

9. The method of claim 8 wherein the source of nitrogen is in an amount of about 3.8 g/l.

10. The method of claim 7 wherein the source of nitrogen is both asparagine and glutamine, and the asparagine is in an amount of about 200 mg/l to about 1 g/l and the glutamine is in an amount of about 500 mg/l to about 2 g/l.

11. The method of claim 10 wherein the asparagine is in an amount of about 500 mg/l and the glutamine is in an amount of about 1 g/l.

12. The method of claim 1 wherein the suspension culture of step (e) has a duration of less than about 20 days.

13. The method of claim 12 wherein the suspension culture of st p (e) has a duration of about 10 days to about 20 days.

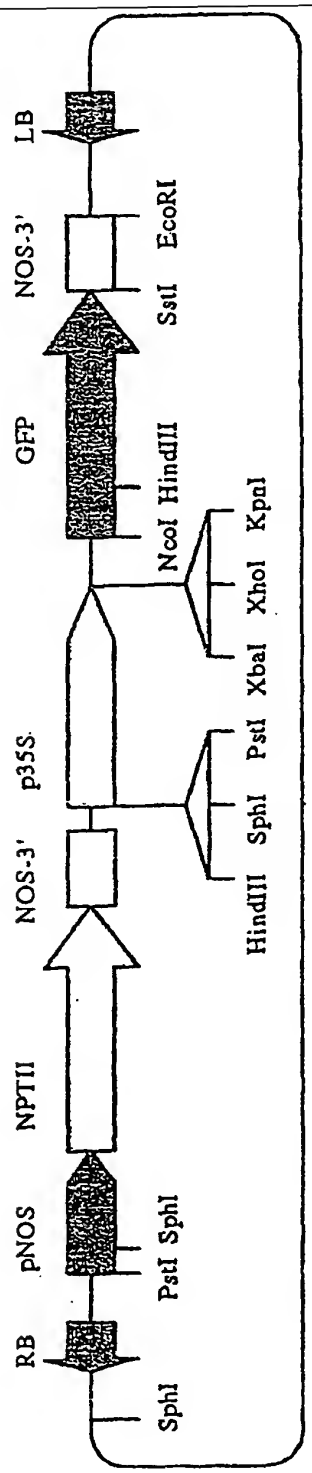
14. The method of claim 13 wherein the suspension culture of step (e) has a duration of about 14 days.

15. The method of claim 1 wherein step (c) is carried out in the presence of low concentration of one or more hormones.

16. The method of claim 15 wherein the concentration of any one hormone ranges from 0 to about 1 mg/l.

17. The method of claim 15 wherein step (c) is carried out in the presence of 2,4-dichlorophenoxacetic acid in a concentration ranging from 0 to about 0.5 mg/l and kinetin in concentration ranging from 0 to about 1 mg/l.

18. The method of claim 17 wherein the 2,4-dichlorophenoxylacetic acid is in a concentration of about 0.05 mg/l and the kinetin is in a concentration of about 0.1 mg/l.


LB
NOS-3'
EcoRI
SstI
GFP
HindIII
NcoI
KpnI
XhoI
XbaI
p35S
PstI
SphI
NOS-3'
HindIII
NPTII
pNOS
PstI
SphI
RB
SphI

PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
GM/MC/R33-68		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/SG 99/ 00058	11/06/1999	
Applicant		
INSTITUTE OF MOLECULAR AGROBIOLOGY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy ot each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble torm.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INT NATIONAL SEARCH REPORT

ernational Application No
T/SG 99/00058

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 12512 A (CALGENE INC) 10 April 1997 (1997-04-10) page 11	1,2,6, 12-14
A	WO 97 43430 A (MAIER CAMELIA G A ;CHAPMAN KENT D (US); HEMPHILL JOHN K (US); UNIV) 20 November 1997 (1997-11-20) the whole document	1-18
A	WO 89 05344 A (PHYTOGEN) 15 June 1989 (1989-06-15) the whole document	1-18
A	EP 0 270 355 A (AGRACETUS) 8 June 1988 (1988-06-08) the whole document	1-18

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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23 February 2000	01/03/2000

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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